

Evidence for *Mycoplasma* spp., *Chlamydia pneumoniae*, and Human Herpes Virus-6 Coinfections in the Blood of Patients With Autistic Spectrum Disorders

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We examined the blood of 48 patients from central and southern California diagnosed with autistic spectrum disorders (ASD) by using forensic polymerase chain reaction and found that a large subset (28/48 or 58.3%) of patients showed evidence of *Mycoplasma* spp. infections compared with two of 45 (4.7%) age-matched control subjects (odds ratio = 13.8, $P < 0.001$). Because ASD patients have a high prevalence of one or more *Mycoplasma* spp. and sometimes show evidence of infections with *Chlamydia pneumoniae*, we examined ASD patients for other infections. Also, the presence of one or more systemic infections may predispose ASD patients to other infections, so we examined the prevalence of *C. pneumoniae* (4/48 or 8.3% positive, odds ratio = 5.6, $P < 0.01$) and human herpes virus-6 (HHV-6, 14/48 or 29.2%, odds ratio = 4.5, $P < 0.01$) coinfections in ASD patients. We found that *Mycoplasma*-positive and -negative ASD patients had similar percentages of *C. pneumoniae* and HHV-6 infections, suggesting that such infections occur independently in ASD patients. Control subjects also had low rates of *C. pneumoniae* (1/48 or 2.1%) and HHV-6 (4/48 or 8.3%) infections, and there were no coinfections in control subjects. The results indicate that a large subset of ASD patients shows evidence of bacterial and/or viral infections (odds ratio = 16.5, $P < 0.001$). The significance of these infections in ASD is discussed in terms of appropriate treatment. © 2007 Wiley-Liss, Inc.

Key words: autism; infection; HHV-6 virus; *Chlamydia pneumoniae*; *Mycoplasma* species

Autism was first identified in 1943 (Kanner, 1943), and autism patients generally suffer from an inability to communicate properly, form relationships with others, and respond appropriately to their environments. Autism patients often display repetitive actions and develop troublesome fixations with specific objects, and they are often sensitive to certain sounds, tastes, and smells. Autism patients do not all share the same signs and symptoms but tend to share certain social, communication, motor, and sensory problems that affect their behavior in

predictable ways (Berney, 2000). Autism and related disorders have been recently placed into a multidisorder category called autistic spectrum disorders (ASD), which includes autism, attention deficit disorder (ADD), attention deficit hyperactivity disorder (ADHD), and other disorders (Keen and Ward, 2004).

The criteria for diagnosis of ASD are, in general terms, the presence of a triad of impairments in social interaction, communication, and imagination (Wing et al., 2002). These signs and symptoms are thought to be due to abnormalities in brain function or structure and are thought to have a genetic basis (Folstein and Rosen-Sheidley, 2001; Veenstra-Vanderweele et al., 2003). The incidence of ASD is currently estimated at 1 in 1,000 children, and in genetically predisposed families the disorder is ~100 times higher in incidence than in the general population (Folstein and Rosen-Sheidley, 2002). The concordance rate in monozygotic twins is 70–90%, whereas in dizygotic twins the rate is close to 0%, suggesting a strong genetic component (Veenstra-Vanderweele et al., 2003).

In some patients, there are also a number of other less specific chronic signs and symptoms. Among these are fatigue, headaches, gastrointestinal and vision problems, and occasional intermittent low-grade fevers and other signs and symptoms that are generally excluded in the diagnosis of ASD. These suggest that a subset of ASD patients may suffer from bacterial or viral infections (Takahashi et al., 2001). There are several reasons for this, including the nonrandom or clustered appearance of ASD, sometimes in immediate family members or particular regions, the presence of certain signs and symptoms associated with infection, the cyclic course of the

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illness, and in some cases its response to antimicrobial therapies.

Although no single underlying cause has been established for ASD, there is growing awareness that ASD can have an infectious nature that may be a cofactor for the illness or appear as an opportunistic infection(s) that can aggravate patient morbidity (Takahashi et al., 2001; Yamashita et al., 2003; Libbey et al., 2005). Identifying systemic infections, such as those produced by *Mycoplasma* species (Huang et al., 1998; Nijs et al., 2002; Nicolson et al., 2003a,b, 2005a), *Chlamydia pneumoniae* (Chia and Chia, 1999; Nicolson et al., 2003a,b), and human herpes virus-6 (HHV-6; Braun et al., 1997; Campadelli-Fiume et al., 1999; Nicolson et al., 2003a,b), is likely to be important in determining the treatment strategies for many ASD patients. These infections can penetrate the CNS and are associated with other neurological diseases (Nicolson et al., 2002). In addition, heavy metal, chemical, and environmental exposures also appear to be important in ASD (Eppright et al., 1996; Colborn, 2004; Davidson et al., 2004).

Here we examined ASD patients to determine whether a subset of patients shows evidence of infection with *Mycoplasma* spp., *C. pneumoniae*, or HHV-6. Because these infections can cause neurological signs and symptoms (Baseman and Tully, 1997; Nasralla et al., 1999, 2000; Nicolson et al., 2003a), they may be important in ASD. Previously we found that children of *Mycoplasma*-positive Gulf War veterans were over 18 times more likely to come down with *Mycoplasma fermentans* than the general population (Nicolson et al., 2003c), and ASD diagnoses were found in their children. In addition, examination of a group of autism patients from civilian families revealed that there was a high incidence of mycoplasmal infections, including *M. fermentans*, *M. pneumoniae*, and *M. hominis* (Nicolson et al., 2005b). Because mycoplasmal infections can often be found as coinfections with *C. pneumoniae* or HHV-6 (Nicolson et al., 2003a,b, 2005a), we examined ASD patients to see whether they had evidence of coinfections of *Mycoplasma* spp., *C. pneumoniae*, and HHV-6.

MATERIALS AND METHODS

Patients

All ASD patients (N = 48) were randomly recruited from families in contact with patient support groups and were referred from central and southern California physicians after diagnosis with ASD according to the *International classification of diseases* (ICD-10) and the *Diagnostic and statistical manual of mental disorders* (DSM-IV). All patients were assessed via the Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1997) and Childhood Autism Rating Scale (CARS; Van Bourgondien et al., 1992; Pilowsky et al., 1998). All of the patients in the study were ASD patients; most (45/48) had a diagnosis of autism, and six of 48 were diagnosed with ADD (three of which were also diagnosed with autism) and nine autism patients with Asperger's syndrome. Patients also underwent a medical history, a sign/symptom illness survey was

taken, and they had routine laboratory tests. Additionally, all parents were questioned about medication use during the 3 months prior to the study, and patients had to be free of antibiotic treatment for 2 months prior to blood collection. Control subjects were from families randomly recruited for unrelated studies (N = 45); they had to be free of any disease or behavioral disorder, and they had not undergone antibiotic treatment for 3 months prior to blood collection.

Blood Collection

Blood was collected in EDTA-containing tubes and immediately brought to ice-bath temperature as described previously (Nijs et al., 2002; Nicolson et al., 2003a-c, 2005a). Samples were shipped with wet ice by overnight air courier to the Institute for Molecular Medicine for analysis. All blood samples were blinded. Whole blood (50 μ l) was used for preparation of DNA using Chelex (Bio-Rad, Hercules, CA), as follows. Blood cells were lysed with nano-pure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13,000g for 2 min, the supernatants were discarded. Chelex solution (200 μ l) was added, and the samples were incubated at 56°C and at 100°C for 15 min each. Aliquots from the centrifuged samples were used immediately for polymerase chain reaction (PCR) or flash frozen and stored at -70°C until use. Multiple aliquots were used for experiments on all patient samples.

Detection of *Mycoplasma* by Forensic PCR

Amplification of the target gene sequences (Nicolson et al., 2003a,b, 2005a) was performed in a total volume of 50 μ l PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9) containing 0.1% Triton X-100; 200 μ M each of dATP, dTTP, dGTP, and dCTP; 100 pmol of each primer; and 0.5–1 μ g of chromosomal DNA. Purified mycoplasmal DNA (0.5–1 ng of DNA) was used as a positive control for amplification. The efficiency of the PCR process was monitored by amplification of β -actin mRNA. Additional primer sets were used to confirm the species specificity of the reaction (Nicolson et al., 2003a-c, 2005a). The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and *M. penetrans*) or 55°C (*M. pneumoniae*, *M. hominis*, *M. fermentans*). Extension temperature was 72°C in all cases. Finally, product extension was performed at 72°C for 10 min. Negative and positive controls were present in each experiment. The amplified samples were run on a 1% agarose gel containing ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). After denaturing and neutralization, Southern blotting was performed as described below.

Chlamydia pneumoniae Detection by Forensic PCR

PCR detection of *Chlamydia* (*Chlamydophila*) *pneumoniae* was performed as described above for various *Mycoplasma* species, except that the conditions and primers differ (Nicolson et al., 2003a,b, 2005a). PCR was carried out with the *C. pneumoniae*-specific primers 5'-TGACAACGTTAGAAA-TACAGC-3' (upstream) and downstream 5'-CGCCTCTC-TCTCCTATAAAT-3'. Additional primer sets were used to

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TABLE I. Patient Demographic Data

	N	Mean age (SD) (years)	Range (years)	Males (%)	Females (%)
Patients	48	8.4 (2.8)	3–14	36 (75)	12 (25)
Controls	45	7.9 (3.3)	4–11	28 (62.2)	17 (37.8)
Rural patients	18	8.1 (2.9)	3–14	14 (77.7)	4 (22.3)
Urban patients	30	8.6 (3.2)	4–14	22 (73.3)	8 (26.7)

AQ1 confirm the species specificity of the reaction. The DNA was amplified for 30 cycles using standard cycle parameters and the product evaluated by agarose gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β -actin mRNA. The presence of amplification inhibitors was evaluated by spiking negative samples. *C. pneumoniae*-specific sequence of the PCR product was confirmed by Southern blot and dot-blot hybridization with a 21-mer internal probe: 5'-CGTTGAGTCAACGACTTAAGG-3' 3'-end-labelled with digoxigenin-UTP or 32 P-labeled probe.

HHV-6 Detection by Forensic PCR

AQ1 PCR detection of HHV-6A was performed as described above, except that the conditions and primers differ, and plasma was used for polynucleotide isolation to detect active infections or cell-released virus (Nicolson et al., 2003a,b). PCRs were carried out using the following HHV-6A-specific primers: 5'-GCGTTTTTCAGTGTGTAGTTCGGCAG-3' (upstream) and 5'-TGGCCGCATTTTCGTACAGATACGGA-GG-3' (downstream). The nucleotides were amplified for 30 cycles with standard cycle parameters, and the product was evaluated by agarose gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β -actin mRNA. The presence of amplification inhibitors was evaluated by spiking negative samples. HHV-6A-specific oligonucleotides in the PCR product were identified by Southern blot and dot-blot hybridization using a 21-mer internal probe: 5'-ATCCGAAACAACACTGTCTGACTGGCA-3' 3'-end-labelled with digoxigenin-UTP or 32 P-labeled probe.

Southern Blot Confirmation

The amplified samples were run on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). After denaturing and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed (Nasralla et al., 1999). Membranes were prehybridized with hybridization buffer consisting of 1 \times Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with digoxigenin-UTP or 32 P-labeled internal probe (10⁷ cpm per bag). After hybridization and washing to remove unbound probe, the membranes were examined (digoxigenin-UTP-labeled probe) or exposed to autoradiography film (32 P-labeled probe) for 0.5–2 days at -70°C (Nicolson et al., 2003a,b, 2005a).

The sensitivity and specificity of the PCR method for detection were determined by examining serial dilutions of purified DNA from the microorganisms in random blood samples. Control DNA samples were provided by the American

Type Culture Collection (Manasses, VA). The primers produced the expected amplification product size in all test species, which was confirmed by hybridization using the appropriate 32 P-labeled internal probe (Nasralla et al., 1999). Amounts as low as a few femtograms of purified DNA were detectable for all species with the specific internal probes. There was no cross-reactivity between the internal probes of one species and the PCR product from another species (Nasralla et al., 2000; Nicolson et al., 2003a–c). The techniques used have been validated in various studies (see, e.g., Berg et al., 1996; Bernet et al., 1995).

Statistical Analysis

Subjects' demographic characteristics were assessed via descriptive statistics and Student's *t*-tests (independent-samples test, *t*-test for equality of means, two-tailed). The 95% confidence interval was chosen for minimal significance. Odds ratios were calculated by using logistic regression (logit method) in Statistica 5.5 (Statsoft, Tulsa, OK). In some cases, Pearson χ^2 test was performed to compare prevalence data between patients and control subjects.

RESULTS

Patients and Control Subjects

ASD patients and control subjects were approximately similar in age (control subjects mean age = 8.4; ASD patients mean age = 7.9). ASD patients differed significantly according to sex distribution ($P < 0.05$); 75% of the patients were male, whereas 25% of the patients were female. Similarly, 62.2% of control subjects were male, whereas 37.8% were female. Patients were from central and southern California and resided in approximately equally in rural and urban environments (Table I).

Bacterial and Viral Infections in ASD Patients

By using PCR, we examined ASD patients' blood for the presence of bacterial and viral infections. Evidence for *Mycoplasma* spp. infections was found in 28 of 48 or 58.3% of ASD patients and two of 45 or 4.7% age-matched control subjects (odds ratio = 13.8, $P < 0.001$). *C. pneumoniae* infections were found in four of 48 or 8.3% of ASD patients and in one of 45 or 2.1% of control subjects (odds ratio = 5.6, $P < 0.01$). We also examined the incidence of HHV-6 infections in ASD patients and found that 14 of 48 or 29.2% of ASD patients were positive compared with four of 45 or 8.8% age-matched control subjects (odds ratio = 4.5, $P < 0.01$). We did not find any multiple coinfections in con-

TABLE II. Prevalence and Odds Ratio Analysis of Infections in ADS Patients and Control

Type of infection	ASD patients N = 48 (%)	Control subjects N = 45 (%)	Odds ratio, P or χ^2
HHV-6	14 (29.2)	4 (8.3)	4.5, $P < 0.01$
<i>C. pneumoniae</i>	4 (8.3)	1 (2.1)	5.6, $P < 0.01$
<i>Mycoplasma</i> spp.	28 (58.3)	2 (4.7)	13.8, $P < 0.001$
<i>M. pneumoniae</i>	16	2	9.2, $P < 0.001$
<i>M. fermentans</i>	17	0	14.8, $P < 0.001$
<i>M. hominis</i>	5	0	11.8, $P < 0.01$
<i>M. penetrans</i>	1	0	6.6, $P < 0.01$
Single mycoplasmal infection	16 (33.3)	2 (4.7)	13.8, $P < 0.001$
Multiple mycoplasmal infections	12 (25.0)	0 (0)	$\chi^2 = 11.7, P < 0.001$
<i>M. fermentans</i> + <i>M. pneumoniae</i>	7	0	$\chi^2 = 4.7, P < 0.01$
<i>M. fermentans</i> + <i>M. hominis</i>	2	0	$\chi^2 = 1.9, P < 0.3$
<i>M. pneumoniae</i> + <i>M. hominis</i>	1	0	$\chi^2 = 1.4, P < 0.2$
<i>M. fermentans</i> + <i>M. hominis</i> + <i>M. pneumoniae</i>	2	0	$\chi^2 = 1.9, P < 0.2$
<i>Mycoplasma</i> + HHV-6	8 (16.7)	0 (0)	$\chi^2 = 4.4, P < 0.01$
<i>Mycoplasma</i> + <i>C. pneumoniae</i>	2 (4.2)	0 (0)	$\chi^2 = 2.1, P < 0.19$
<i>C. pneumoniae</i> + HHV-6	1 (2.1)	0 (0)	$\chi^2 = 1.6, P < 0.3$

T2 trol subjects (Table II). The rate of positive results in control subjects was similar to that in previous studies (Nasralla et al., 2000; Nicolson et al., 2003a–c, 2005). The differences between infections in ASD patients and control subjects were highly significant (odds ratio = 16.5, $P < 0.001$). Significant differences were not found in the prevalence of infections between urban and rural patients, between male and female patients, or between autism and other ASD diagnoses.

Multiple Coinfections in ASD Patients

We studied multiple infections in patients by examining whether patients who were positive (or negative) for one type of infection also tested positive for other infections. Eight of fourteen patients with HHV-6 positive results (57.1%) were also positive for mycoplasmal infections. *C. pneumoniae* infections were observed in two of four mycoplasma-positive ASD patients and two of four mycoplasma-negative patients. Therefore, we did not find a preference for particular multiple infections in patient subsets. Multiple mycoplasmal infections were found in 12 of 48 or 25% of ASD patients; only *M. fermentans* plus other species were found. We examined 45 control subjects who did not show clinical signs and symptoms and found that only two were positive for a single mycoplasma species (*Mycoplasma pneumoniae*; Table II). Differences between ASD patients and control subjects were highly significant (Table II).

DISCUSSION

Previously we found that chronic infections in Gulf War veterans diagnosed with Gulf War illness could also be found in symptomatic family members, including their children (Nicolson et al., 2003c). In the families chosen for this study, chronic illnesses were not reported until after the veteran in the family returned from the Gulf War. Interestingly, though not reported, common diagnoses of illness in the children of Gulf War veterans

with mycoplasmal infections included ASD-like illnesses, among others, and we found the same infection, primarily *M. fermentans*, in both the sick adults and the children in these families. The data suggested that the *M. fermentans* was likely passed from the veterans to their children (Nicolson et al., 2003c). Although preliminary and not carefully analyzed or studied further, this result suggested that infections might be present in ASD patients. Therefore, we examined a small group of patients (28 patients with autism-like disorders, age range 3–12 years) in central California for evidence of mycoplasmal infections, and we found that slightly over one-half were positive for one of four species of *Mycoplasma* (Nicolson et al., 2005b). In contrast to the children in military families among whom primarily one species of *Mycoplasma* was found (usually *M. fermentans*), most ASD patients in central California were found to have single or multiple mycoplasmal infections involving *M. pneumoniae*, *M. fermentans*, *M. hominis*, or *M. genitalium*. We found similar results in the present study, but, in addition to infections with *Mycoplasma* spp., we also examined two other commonly found infections in chronically ill patients, *C. pneumoniae* and HHV-6 (Nicolson et al., 2003a,b). The results suggested that infections are a common feature in ASD. Consistent with this hypothesis is the finding that autism occurs at greater prevalence during periods of more frequent hospitalizations for bronchitis or pneumonia (Tanoue et al., 1988), and maternal viral infections during the second trimester of pregnancy are associated with increased risk of autism in the offspring (Ciaranello and Ciaranello, 1995; Wilkerson et al., 2002). Infections are thought to play important roles in a variety of neurodevelopmental diseases, including ASD (Horning et al., 1999; Nicolson et al., 2002; Libbey et al., 2005). Such infections could be involved in the etiology of the disease, or more likely they could cause comorbid states (Nicolson et al., 2003a,b, 2005).

We found a higher prevalence of *Mycoplasma* spp. (odds ratio = 13.8), *C. pneumoniae* (odds ratio = 5.6),

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and HHV-6 (odds ratio = 4.5) among children diagnosed with ASD compared with age-matched control subjects. The PCR techniques used in the present study have been validated in other studies (Nicolson et al., 2003a–c, 2005). There are some similarities between the environmental exposures of Gulf War veterans and children with ASD. Both groups were given multiple vaccines prior to their illnesses, and heavy metals and chemicals have been found in both groups (Eppright et al., 1996; Boyd, 2004; Buttram, 2004; Davidson et al., 2004; Geier and Geier, 2004), but these findings are not universal (Jackson and Garrod, 1978). There are reports of clinical improvement with treatment for these environmental exposures (for review see Kidd, 2002).

There were limitations in the present preliminary study, including sample size. Although all of the patients in the study were ASD patients, most (39/48) had a diagnosis of autism, and six of 48 were diagnosed with Asperger's syndrome, whereas six of 48 were diagnosed with ADD (three of whom were also diagnosed with autism). Removal of the two sets of six patients from the analysis or analysis of the data by sex did not change the results or conclusions. Other factors, such as geography, family socioeconomic status, vaccination records, and family educational levels were not analyzed. There were also limitations in the diagnostic tests performed on patients. Future studies should include additional tests on the patients' intellectual capacities with regard to abstracting and generalization as well as verbal and nonverbal communications.

The infections found in ASD patients in the present and previous studies (Takahashi et al., 2001; Yamashita et al., 2003; Libbey et al., 2003; Nicolson et al., 2003c, 2005) could have originated from vaccines or from opportunistic infections in immune-suppressed children. Bacterial contamination has been found in commercial vaccines, and in one study 6% of commercial vaccines were contaminated with mycoplasmas (Thornton, 1986). Thus the appearance of infections in children diagnosed with ASD may eventually be linked to the multiple vaccines received during childhood either as a source or from opportunistic infections in immune-suppressed recipients of multiple vaccines. Although the etiology of ASD is currently unknown and is thought to involve both genetic and environmental factors (Lipkin and Hornig, 2003; Libbey et al., 2005), the infections found in ASD patients should be considered along with other factors in the management of these disorders (Kidd, 2002).

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